[CANCER RESEARCH 44, 4254–4259, October 1984]

In Vivo Dosimetry of 4-Aminobiphenyl in Rats via a Cysteine Adduct in Hemoglobin[1]

Laura C. Green, Paul L. Skipper, Robert J. Turesky, Matthew S. Bryant, and Steven R. Tannenbaum[2]

Massachusetts Institute of Technology, Department of Nutrition and Food Science, Cambridge, Massachusetts 02139

ABSTRACT

The feasibility of monitoring doses of 4-aminobiphenyl (ABP) via adduction to hemoglobin was investigated. Rats dosed with ABP (from 0.5 μg/kg to 5 mg/kg) formed a stable covalent hemoglobin:ABP adduct. Approximately 5% of a single dose was bound as hemoglobin:ABP; chronic dosing led to an accumulation of the adduct to a level 30 times greater than that found after a single dose. Facile *in vitro* hydrolysis of the adduct regenerated ABP, allowing detection at the sub-ng level. Human hemoglobin was also readily adducted, using *N*-hydroxy-ABP *in vitro*. The predominant site of adduction appeared to be the cysteine residue in hemoglobin. The use of such adducts as dosimeters for arylamine exposures in humans is discussed.

INTRODUCTION

In order to assess the risks to human health posed by carcinogens in the environment, one must estimate the actual doses of these compounds. In the case of a food-borne carcinogen, for example, one can estimate intakes by measuring levels in various foods. A more accurate measure of dose, however, might be provided by the measurement of the compound or one of its metabolites in exposed individuals. The levels in food only provide estimates of exposure, while the levels *in vivo* would serve as dosimeters, reflecting actual absorption and metabolism.

One type of dosimetry involves simply measuring amounts of a given compound in human tissues. Pesticides and polychlorinated biphenyls, for example, can be measured in blood or adipose tissue (4). Measurements such as these will be most useful when the compounds have long biological lifetimes, as is, of course, the case with the chlorinated pesticides.

More often, though, the compounds of interest will be rapidly eliminated and/or metabolized. In these cases, the compounds may not be detectable *per se* but may accumulate as covalent adducts to macromolecules, such as nucleic acids and proteins. Bunn *et al.* (9, 10) and others (6, 25, 38) have found that glucose, for example, adducts to hemoglobin, forming the glycosylated Hb A_{1c}.[3] Hb A_{1c} levels accumulate over the 4-month lifetime of the protein, and clinical measurements of the adduct provide more reliable and more sensitive assessments of glucose status in diabetic individuals than do the simple measurements of free glucose in blood or urine (31).

The use of hemoglobin adducts as dosimeters for alkylating agents was first investigated by Ehrenberg *et al.* (14) for the mutagen ethylene oxide. These investigators showed that the adducts formed in mice in a dose-dependent fashion accumulated over the lifetime of the erythrocyte during chronic exposures and might be used as a dosimeter in people exposed to ethylene oxide in the workplace (11, 15, 34). Others have begun investigations on the adduction of hemoglobin by methylating agents and by a variety of carcinogens (16, 32, 36).

This report presents our initial work aimed at developing a dosimeter for ABP. This compound is of considerable toxicological significance. It is recognized to be a human bladder carcinogen (20, 21) and is present at levels of a few ng per cigarette in mainstream smoke and greater than 100 ng per cigarette in sidestream smoke (35). The increased risk of cigarette smokers for bladder cancer (12, 28, 44) has been attributed to the presence of carcinogenic arylamines, such as ABP, in cigarette smoke (27). Furthermore, it appears that bladder cancer risk is, in part, dependent upon interspecies (22, 29) and interindividual (27) differences in metabolism of aromatic amines, specifically in the relative rates of *N*-hydroxylation and *N*-acetylation. Dosimetry reflective of the yield of relevant metabolites might therefore be especially useful. We report here that an unusually high percentage of administered ABP forms adducts to hemoglobin, suggesting that this method could be used to determine actual doses in cigarette smokers and other exposed individuals.

MATERIALS AND METHODS

In Vivo Experiments. Young adult male Fischer rats (Charles River Breeding Laboratories) with an average weight of 200 g were given i.p. injections of either ABP (Sigma) or [2,2'-^{3}H]ABP (Midwest Research Institute; found to be >91% radiochemically pure by thin-layer chromatography and diluted to a specific activity of 30 mCi/mmol with unlabeled ABP) in dimethyl sulfoxide. Doses ranged from 0.5 μg/kg to 5 mg/kg. Two or 3 rats were dosed at each level. For single-dose studies, blood was obtained by cardiac puncture or via the abdominal aorta at 24 or 48 hr after dosing. For multiple-dose studies, blood was obtained by puncture of a tail vein. The blood was pooled and iced immediately and centrifuged to generate packed RBC. The RBC were washed 3 times in several volumes of cold phosphate-buffered saline and then lysed by the addition of 3 volumes of cold distilled water. Four volumes of 0.67 M phosphate, pH 6.5, were added to the lysate to effect complete solution of the hemoglobin (8, 39), and the hemolysate was centrifuged at 25,000 × *g* at 4° for 25 min to precipitate the cell membranes and any residual WBC. A small portion of the supernatant was reserved for Protosol (New England Nuclear) digestion, H_2O_2 bleaching, and liquid scintillation counting, while the bulk of the supernatant was dialyzed overnight at 4° against distilled deionized water. In most cases, the remainder of the work-up was as follows. After reserving a sample for LSC, the dialysate was added dropwise to 20 volumes of iced 1% HCl in acetone; this separates the heme, which remains in solution, from the globin, which precipitates (1). The acetone was evaporated from the acidic acetone solution under an N_2 stream, and the reddish-brown residue was either analyzed by LSC or further purified on a C_{18} Sep-Pak (Waters Associates). Three fractions were collected from the Sep-Pak: material that eluted with (*a*) 10 mM KCl buffer, pH 2.5; (*b*) buffer:methanol (1:1); and

[1] This investigation was supported by Grant 2-PO1-ES00597-13 awarded by the National Institute of Environmental Health Sciences.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: ABP, 4-aminobiphenyl; *N*-OH-ABP, 4-hydroxylaminobiphenyl; Mb, myoglobin (in combination); Hb, hemoglobin (in combination); LSC, liquid scintillation counting; GC, gas chromatography; HPLC, high-pressure liquid chromatography; MetMb, metmyoglobin; MetHb, methemoglobin.

Received November 21, 1983; accepted June 27, 1984.

c) methanol. The eluates were concentrated by rotary evaporation as needed and analyzed by LSC and/or HPLC, as below. The globin precipitate was washed once with cold acidic acetone, twice with cold acetone, collected on filter paper in a Buchner funnel, and dried by aspiration, and a sample was analyzed by LSC.

When the samples were to be analyzed by GC, a different work-up was used. A complete description of the procedure will be presented elsewhere, but the essential features are the following. The hemolysate was spiked with 1.0 ng of 4'-fluoro-ABP to serve as internal standard and then made 0.1 M in NaOH. Base-catalyzed hydrolysis was found to be preferable to acid-catalyzed hydrolysis, since it yielded a much cleaner chromatogram. After incubation at 37° for 2 hr, the mixture was extracted twice with 0.5 volume of hexane. Pentafluoropropionic anhydride (1 µl) was added to the hexane after it was dried over Na_2SO_4. After 10 min, the hexane solution was concentrated with a rotary evaporator to 0.2 to 0.5 ml for analysis by GC. The recovery of internal standard was typically about 40%. It should be emphasized that purity of solvents and reagents is critical to the success of this method and that only glass, stainless steel, and Teflon apparatus should be used.

***In Vitro* Experiments.** Sperm whale myoglobin (Sigma Chemical) was prepared as metMb as follows: 1.2 mol of $K_3Fe(CN)_6$ were reacted with 1 mol of myoglobin in 50 mM sodium phosphate, pH 7.0, for 2 min. The solution was then passed through a Bio-Gel P-6DG size exclusion column, equilibrated previously in the same buffer, to separate the oxidant from the protein. O_2Mb was prepared by adding a few crystals of sodium dithionite to a solution of Mb in 50 mM phosphate, pH 7.0, which had been flushed with N_2; the mixture was then passed through the Bio-Gel column.

Hemoglobin was prepared from human blood obtained by venipuncture and collected in heparinized Vacutainers. Red cells were harvested and washed as above and then lysed with an equal volume of cold distilled water and 0.1 volume of toluene. The mixture was shaken vigorously for 1 min, allowed to sit on ice for 1 hr, and then centrifuged at 20,000 × *g* for 30 min. The Hb-containing supernatant was further purified by passing it through the Bio-Gel P-6DG column. Samples were analyzed by visible spectroscopy to confirm that all of the Hb existed as O_2Hb. MetHb was prepared by oxidation with 1.2 mol of $K_3Fe(CN)_6$ per mol of heme, followed by purification through Bio-Gel P-6DG.

Concentrations of hemoglobin or myoglobin were determined by oxidation with $K_3Fe(CN)_6$, complexation with NaCN, and measurement of the absorbance at 540 nm (41).

Reactions of N-[2,2'-^{3}H]OH-ABP [synthesized by the method of Thissens *et al.* (40); specific activity, 4.1 mCi/mmol] with O_2Mb, O_2Hb, or metHb proceeded at room temperature for 1 hr and were monitored by visible spectroscopy. Cysteine residues available before and after the reaction were determined by titration with *p*-mercuribenzoate (5, 7).

In experiments designed to determine the nature of the protein adducts formed, N-[2,2'-^{3}H]OH-ABP was allowed to react with solutions of myoglobin or hemoglobin in ratios of 1 mol of N-[2,2'-^{3}H]OH-ABP to 2 mol of heme. Reaction mixtures were passed through Bio-Gel P-6DG, samples were analyzed by LSC, and the solutions of hemoprotein were treated with cold acidic acetone and further purified as above.

In control experiments, [^{3}H]ABP was added to a solution of O_2Hb, in a ratio of 1 mol of ABP to 2 mol of heme. UV-visible spectra taken of samples of the mixture following a 1-hr incubation at room temperature revealed no detectable oxidation of O_2Hb. The mixture was passed through Bio-Gel P-6DG as above; 99.8% of the applied tritium was removed by the column, *i.e.*, only 0.2% of the tritium eluted with the fraction that contained the hemoglobin. This result suggests that chromatography on Bio-Gel is sufficient to dissociate unreacted, noncovalently bound ABP from hemoglobin.

Chromatographic Analysis. HPLC was performed on a C_{18}-µBondapak column (Waters Associates), with a solvent flow rate of 1.5 ml/min and, generally, either of 2 sets of solvents and gradients: (*a*) an acidic buffer as the weak solvent (10 mM KCl, pH 2.5) and methanol as the strong solvent, with a linear gradient from 0% methanol to 80% methanol in 25 min; or (*b*) a neutral buffer of 10 mM K_2HPO_4/KH_2PO_4, pH 7, as the weak solvent and methanol as the strong solvent, with a linear gradient from 20% methanol to 80% methanol in 30 min. Detection was either by UV absorption at 254 nm (Model 440 Absorbance Detector, Waters Associates) or by fluorescence (Schoeffel FS970 fluorimeter). For fluorescence, excitation was at 247 nm under acidic conditions, excitation was at 280 nm under neutral conditions, and emission was through a 320-nm cutoff filter in both cases. For some analyses, HPLC effluent was monitored by a continuous scanning spectrophotometer (Model 8450, Hewlett-Packard). Radioactivity was monitored by collecting successive 1.5-ml aliquots of the HPLC effluent and analyzing by LSC.

GC was performed with a Hewlett-Packard 5880 chromatograph equipped with a capillary inlet and an electron-capture detector. The column was a 15-m x 0.25-mm ID fused silica capillary coated with Supelcowax 10 (film thickness, 0.25 µm). The carrier and make-up gases were hydrogen and 5% methane in argon, respectively (Matheson UHP grade). Injections were made in a splitless mode with the port temperature maintained at 300°. Initial oven temperature was 100° for 1 min, followed by 2 temperature programs (100–170° at 30°/min and 170–210° at 5°/min), followed by an isothermal phase.

RESULTS

Rats dosed with [^{3}H]ABP accumulated a considerable fraction of the dose in their blood. Twenty-four hr after a single dose of 5 mg/kg, rats showed 8.0% of the administered radioactivity in the RBC compartment and less than 10% of this in the plasma compartment. After dialysis of the washed, lysed, membrane-free RBC, 7.3% of the dose remained. After treatment with acidic acetone, only 0.13% of the dose precipitated with the globin, while 6.6% of the dose was recovered in the acidic acetone solution. When this solution was evaporated and further purified on a C_{18}-Sep-pak, 5.6% of the dose eluted in the 50:50 buffer:methanol wash. Chromatography of this eluate revealed that the major labeled product was [^{3}H]ABP itself, accounting for 5% of the original dose (Chart 1). Since both prolonged dialysis of the RBC lysate and treatment of the hemolysate with acetone alone, in the absence of acid, failed to liberate significant radioactivity, it appeared that a form of ABP had covalently bound to hemoglobin *in vivo* and that this adduct was acid hydrolyzed *in vitro*.

In vivo, the level of the acid-labile hemoglobin adduct formed was directly proportional to the dose of ABP, over the range of doses administered (Chart 2).

These experiments, which were performed with radioactive ABP, may actually underestimate the amount of acid-labile adduct, as indicated by experiments in which this adduct was quantified by GC, a method which incorporates an internal standard to compensate for losses in sample handling. Four animals were treated with ABP at 0.5 µg/kg. GC quantification of the releasable ABP yielded an average value of 0.040 ± 0.017 (S.E.) nmol/g of hemoglobin, which corresponds to 13% of the dose bound to the total Hb compartment. These data are not included in Chart 2. The value of 13% needs to be adjusted downward to about 11%, however, because there is a background which appears in the absence of an administered dose. The source of this background is uncertain, but it has been observed in 13 of 13 animals examined thus far. In 8 cases, an accurate quantification was made, giving a mean value of 0.006 ± 0.002 nmol/g of hemoglobin. Control experiments demonstrated that the compound giving rise to the background is a basic compound, since it, as well as the internal standard, could be extracted out of the hexane extract with acid. Thus, it seems likely that the background is indeed ABP.

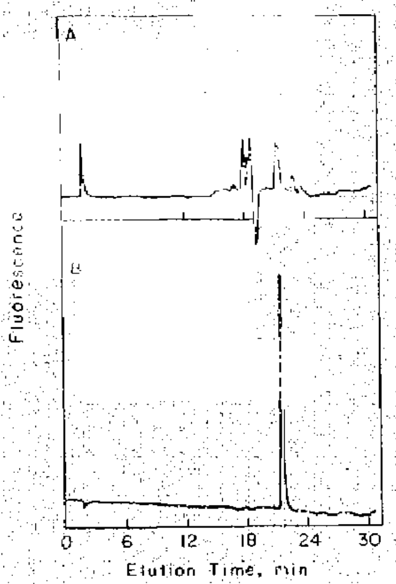

Chart 1. High-pressure liquid chromatograms of *in vivo* sample (*A*) and standard (*B*), with fluorescence detection. Standard is 7 ng of ABP in 0.05 N HCl. Sample is 0.5 ml of the 50:50 buffer:methanol wash of the preparation of dosed rat blood (see text for details). Chromatography was under acidic conditions, as described in the text. The HPLC eluate was collected in 1-min fractions for liquid scintillation counting. Greater than 90% of the applied radioactivity was found to have eluted between 21 and 23 min.

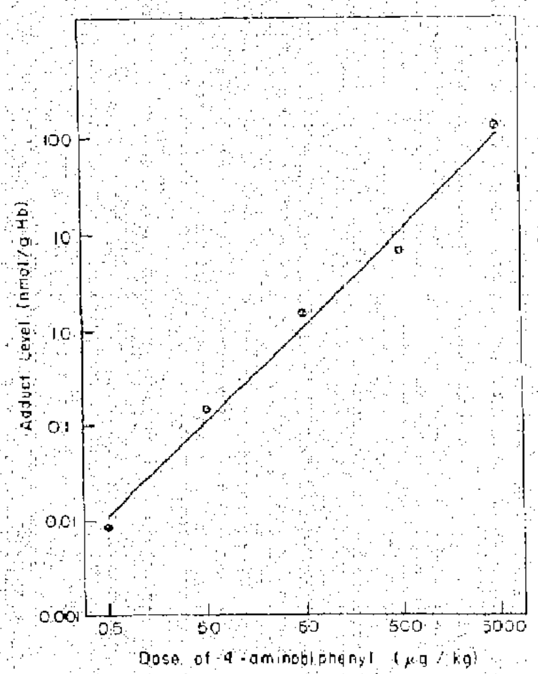

Chart 2. *In vivo* formation of the stable Hb:ABP adduct as a function of single i.p. doses of [^{3}H]ABP. Adduct levels were measured by hydrolysis *in vitro* to free [^{3}H]ABP.

Chronic administration of ABP (11.4 μg of [^{3}H]ABP given once every 48 hr; specific activity, 0.18 μCi/μg) to a single rat led to an accumulation of radioactivity in the blood that was some 30 times greater than that found after a single dose (Chart 3). Upon cessation of dosing, the level of bound radioactivity decreased

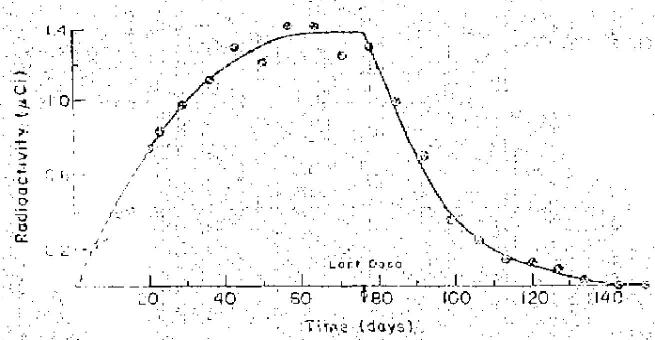

Chart 3. Accumulation of radioactivity in the total blood volume of a rat dosed by gavage with 11.4 μg of [^{3}H]ABP (2.05 μCi) in corn oil once every 2 days for 75 days. The rat weighed 200 g on Day 1 of the experiment, and 550 g by Day 150. The total blood compartment was calculated by assuming that a rat's blood volume is equal to 6.4% of its body weight (average of values reported in Ref. 3).

Table 1

Binding of N-[2,2'-^{3}H]OH-ABP to hemoglobin and myoglobin in vitro

N-[2,2'-^{3}H]OH-ABP, 0.05 mol, was reacted per mol of heme in 2% acetonitrile (to solubilize the hydroxylamine):98% 50 mM phosphate buffer, pH 7. Values shown are the average of duplicates which differed from each other by no more than 10%.

Hemoprotein	% of ^{3}H bound to protein
Oxyhemoglobin	41.6
MetHb	44.3
Oxymyoglobin	6.4
MetMb	5.9

by 2.5% of its peak value per day. This suggested that the adduct was cleared somewhat more rapidly than unmodified hemoglobin, which is removed at a rate of 1.7% per day (37).

To investigate the chemistry of Hb:ABP adduct formation, we reacted N-OH-ABP with hemoglobin or myoglobin *in vitro*; the hydroxylamine was used since it is a major initial metabolite (29). As shown in Table 1, hemoglobin bound some 7 times more N-[2,2'-^{3}H]OH-ABP than did myoglobin, on a per mol globin basis; of the radioactivity bound to hemoglobin, 85 to 90% was recovered as free [^{3}H]ABP upon acid hydrolysis. The identity of the hydrolysis product was based on its cochromatography with authentic ABP and spectral characterization of the chromatographically purified product. The UV spectra under acidic (50% methanol, 50% 0.1 N HCl) and neutral (50% methanol, 50% 0.05 M ammonium formate) conditions and the mass spectrum (Chart 4) were identical to those displayed by authentic ABP.

The difference in adduct yields between hemoglobin and myoglobin suggested that cysteine might be an important site for binding, since sperm whale myoglobin has no cysteine residues (2). Titration of the reaction mixtures of N-OH-ABP and O_2Hb or metHb with *p*-mercuribenzoate in fact revealed that the adduction of N-OH-ABP to hemoglobin involved the proportional loss of available sulfhydryl residues (Chart 5). Visible spectroscopy also showed that the reaction of the hydroxylamine with O_2Hb or O_2Mb involved oxidation to the met- form, as expected (23).

DISCUSSION

The *in vivo* association of aromatic amines with the RBC and with hemoglobin has been demonstrated by several investigators. As early as 1945, Miller *et al.* (30) found that rats dosed with 4-aminoazobenzene produced RBC that liberated the compound upon treatment with base in alcohol. In 1960, Goldblatt *et al.* (17) reported that animals dosed with ^{14}C-labeled 2-

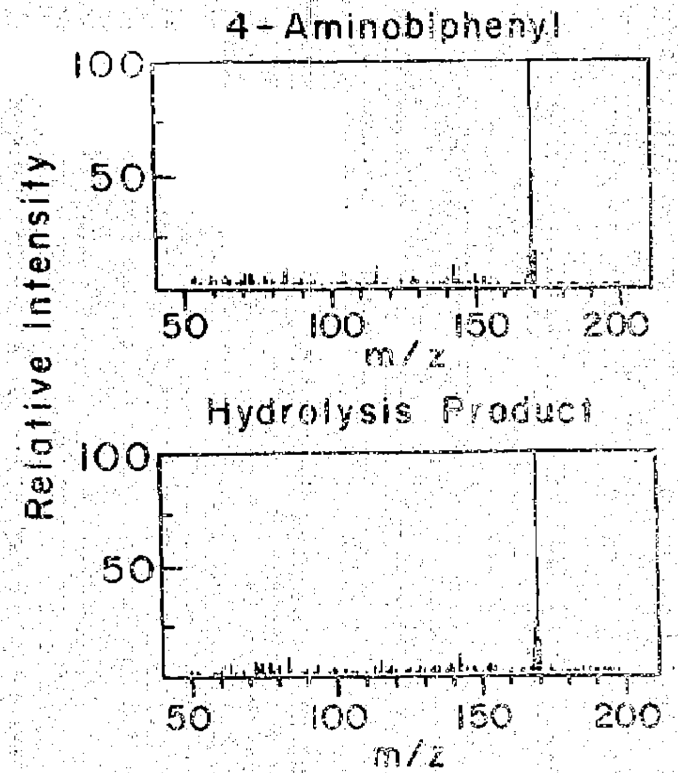

Chart 4. Mass spectra of authentic ABP and the product obtained from hydrolysis of adducted hemoglobin. Spectra were acquired with a Hewlett-Packard 5995 GC-MS system using a Hewlett-Packard cross-linked methyl silicone-fused silica capillary column. Identical retention times were observed for each compound.

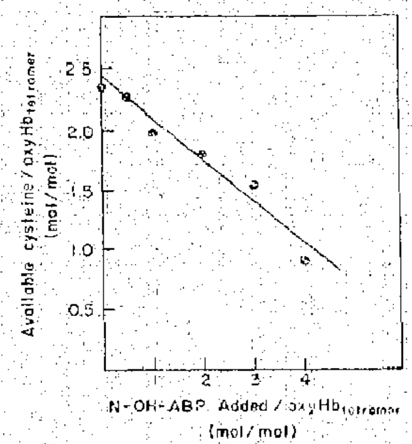

Chart 5. Loss of available cysteine in oxyhemoglobin as a function of N-OH-ABP added *in vitro*. Cysteine residues were determined by titration with p-mercuribenzoate.

naphthylamine accumulated a fraction of the radioactivity that was "strongly attached" to one or more constituents of the contents (as opposed to the membrane) of the RBC. Lotlikar et al. (26) found that 2-nitrosofluorene but not *N*-hydroxy-2-aminofluorene reacted *in vitro* with glutathione to yield a water-insoluble product. Treatment of this 2-nitrosofluorene-glutathione derivative with acid, base, or liver homogenate regenerated the parent 2-aminofluorene. Weisburger *et al.* (42) reported that rats dosed with *N*-acetyl-2-aminofluorene or the *N*-hydroxy derivative localized a portion of the dose within the RBC in a tightly bound form, as judged by resistance to extraction by organic solvents; 24 hr after dosing, 1.5% of the administered N-OH-acetyl-2-aminofluorene was so bound. Gutmann *et al.* (19) found that liver proteins of rats given N-acetyl-2-aminofluorine were also adducted, apparently *via* reaction of the 2-nitroso-derivative. Dolle et al. (13), Groth and Neumann (18), Neumann et al. (33), and Wieland and Neumann (43) have shown that intermediates originating during metHb formation from *trans*-4-aminostilbene and related aromatic amines covalently bind to hemoglobin, that the hemoglobin-binding proclivities of the aromatic amines depend both on their metHb-forming activity and on other measures of their acute and chronic toxicities, and that the reaction of glutathione and protein cysteine residues with aromatic nitroso-derivatives include the formation of an acid-labile sulfinamide adduct. Finally, Kiese and Taeger (24) have shown that hemoglobin cysteine residues are adduced by phenylhydroxylamine *in vitro*. The overall mechanism, then, by which the Hb:ABP adducts may be formed *in vivo* and hydrolyzed *in vitro* is depicted in Chart 6. The arylamine is first oxidized to the arylhydroxylamine, predominantly by the action of cytochrome P-450 in the liver; the arylhydroxylamine then reacts with oxyhemoglobin in a cooxidation, yielding the nitrosoaromatic compound and methemoglobin (23). Nitrosobiphenyl would be expected to react with nucleophiles, and our results suggest that cysteine residues on Hb are the major site of aminobiphenyl adduction. The reaction product of nitrosobiphenyl and the cysteine residue would be expected to rearrange to the sulfinamide, as shown. This adduct would be labile to acid or base hydrolysis *in vitro*, yielding the sulfinic acid and regenerating the original arylamine.

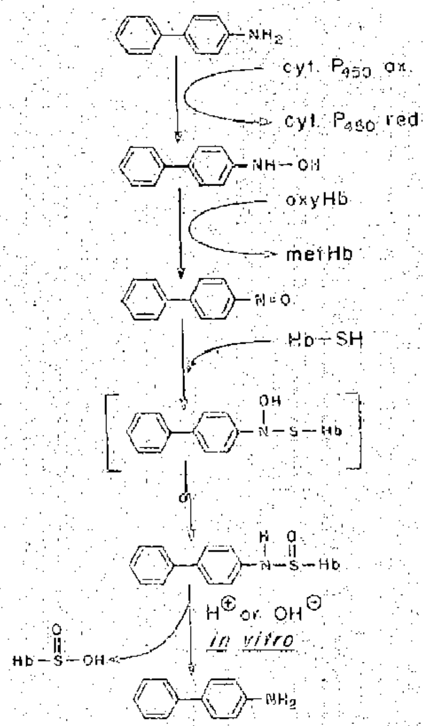

Chart 6. Scheme for ABP:Hb adduct formation and hydrolysis.

While we have yet to prove this sequence of reactions or the

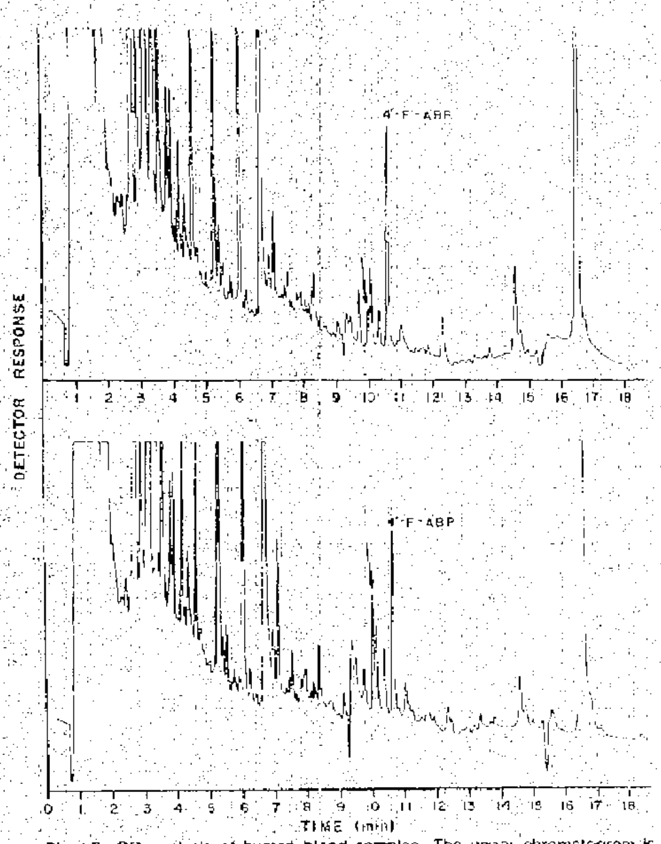

Chart 7. GC analysis of human blood samples. The upper chromatogram is from an untreated sample. The sample used for the lower chromatogram was obtained from the same individual at the same time and spiked with ABP-adducted hemoglobin containing 0.3 ng of ABP. The 2 *peaks* marked with *arrows* have the same retention time as authentic ABP. The amount of internal standard [4'-fluoro-4-aminobiphenyl (4'-F-ABP)] was 1.0 ng. The peak corresponding to ABP in the unspiked sample represents 0.15 ng, as judged by the internal standard.

structure of the Hb-ABP adduct, such proof is not necessary in exploiting the adduct as a dosimeter; its lability to hydrolysis *in vitro* reduces the problem of analysis to that of simply detecting ABP. The question is, then, are available technologies for the detection and quantification of aromatic amines sufficient for the levels anticipated to arise from possible or probable exposures? Before answering this question, we must address the corollary question of what adduct levels to anticipate.

The amount of ABP that might be generated from a 10-ml blood sample taken from a 2 pack-per-day smoker can be estimated as:

$$(40 \text{ cigarettes/day} \times 2 \text{ ng of ABP/ cigarette}) \times 60 \text{ days} \times 0.05 \times 0.002 = 0.5 \text{ ng of ABP}$$

where (*a*) 60 days is one-half of the lifetime of the human RBC and is therefore the multiplier to be used to calculate the steady-state level (34); (*b*) 0.05 represents the fraction of administered ABP which, in the rat, forms a stable, hydrolyzable hemoglobin adduct; and (*c*) 0.002 is the fraction of an adult's entire blood volume represented by 10 ml.

Chart 7 illustrates an experiment designed to demonstrate the adequacy of present technology to measure such levels. Blood was obtained from a volunteer nonsmoker. One sample (10 ml) was worked up as described for GC analysis. Another sample (also 10 ml) was spiked with hemoglobin obtained from a rat dosed with [^{3}H]ABP and estimated to contain 0.3 ng of adducted ABP by LSC. It was then worked up exactly as the first sample. Estimation of the ABP content by comparison to the internal standard yielded a value of 0.4 ng after correction for the apparent background. The difference is probably not significant. It is clear from this experiment that levels of ~0.05 ng/10 ml blood are detectable and should be accurately quantifiable with only slight improvements in chromatography.

Compared to the hemoglobin binding levels found for other carcinogens (36), the percentage of ABP bound as Hb:ABP is quite large. The high yield is probably due to the metabolic action of hemoglobin in converting the hydroxylamine to the reactive nitroso derivative. One would expect high yields of hemoglobin adducts for arylamines in general, as shown by some of the investigations cited above. Since some of these compounds, such as benzidine and 2-naphthylamine, are also human carcinogens (21), it would be of interest to develop the analogous dosimetries.

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